UNIVERSITI PUTRA MALAYSIA

SEROPREVALENCE OF LEPTOSPIROSIS AND BRUCELLOSIS IN LONG-TAILED MAC AQUES *(MACACA FASCICULARIS)* OF PENINSULAR MALAYSIA

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SEROPREVALENCE OF LEPTOSPIROSIS AND BRUCELLOSIS IN LONG-TAILED MAC AQUES *(MACACA FASCICULARIS)* OF PENINSULAR MALAYSIA

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It is hereby certified that I/we* have read this project paper entitled "Seroprevalence of Leptospirosis and Brucellosis in Long-tailed macaques (*Macaca fascicularis*) of Peninsular Malaysia', by Yong Suit-B, Chyna and in my/our* opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of the requirement for the course VPD 4901 – Final Year Project.

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DEDICATION

I dedicate this dissertation to

My mother who has been a constant support and encouragement for everything I choose to pursue. Her unconditional love and dedication is irreplaceable and she is an inspiration for me to always do my best. She is precious and I am grateful to have a superwoman like her every day of my life.

My four-legged best friend, Twinkle, who has been a treasured companion for the past 15 years. No words can describe how she continues to inspire me to carry on with this veterinary course. I will always remember the silent intimate moments we share, how she makes me smile through challenging times and cherish all the time she has left in this world.

Nature and her beautiful residents.

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ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4901 – Projek Ilmiah Tahun Akhir.

SEROPREVALENS *LEPTOSPIROSIS* DAN *BRUCELLOSIS* PADA KERA KETAM (*MACACA FASCICULARIS*) DI SEMENANJUNG MALAYSIA

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2017

Penyelia: Prof Madya Dr. Latiffah Hassan

Penyakit *leptospirosis* dan *brucellosis* adalah penyakit zoonotik yang penting di seluruh dunia dengan insiden yang tinggi di negara tropika yang menjejaskan biodiversiti, kesihatan manusia dan haiwan, kebajikan haiwan dan ekonomi (OIE, 2014; WHO, 2011). Populasi manusia yang semakin berkembang dan pembandaran yang pesat telah menyebabkan peningkatan interaksi hidupan liar dan manusia. Di Malaysia, penambahan konflik antara manusia dan kera (Hambali, 2012) meningkatkan risiko jangkitan penyakit. Objektif kajian ini adalah untuk menentukan seroprevalens *leptospirosis* dan *brucellosis* pada kera ketam Semenanjung Malaysia. Seratus sampel serum telah diuji untuk antibodi terhadap *leptospirosis* dan *brucellosis* dengan mengunakan Microscopic Agglutination Test (MAT) dan Rose Bengal Plate Test (RBPT) masing-masing. Empat belas peratus (14/100) didapati positif untuk *leptospirosis*. Serovar yang paling lazim dikenalpasti

adalah Cellodoni (4%) dan Pyrogenes (4%), diikuti Icterohaemorrhagiae (3%), Bataviae (2%) dan Lai (1%). Seroprevalens *leptospirosis* pada kera jantan adalah lebih tinggi dibandingan kera betina. Kera jantan adalah 4.5 kali lebih mungkin seropositif dibandingkan dengan kera betina. Ini mencadangkan bahawa perbezaan tingkah laku jantina mempengaruhi pendedahan kepada *leptospirosis*. Tiada perbezaan didapati antara seroprevalens dengan umur, habitat dan kawasan. Semua sampel adalah seronegatif terhadap *brucellosis*. Kajian ini menunjukkan bahawa kera ketam terjangkit *leptospirosis* menimbulkan risiko kesihatan awam kerana boleh berlakunya penyebaran silang spesis.

Kata Kunci: *Leptospirosis, Brucellosis, Microscopic agglutination test (MAT), Rose Bengal Plate test (RBPT), Zoonotik, Kera ketam (Macaca fascicularis), Primata, Semenanjung Malaysia*

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary medicine in partial fulfilment of the course VPD 4901– Final Year Project.

SEROPREVALENCE OF LEPTOSPIROSIS AND BRUCELLOSIS IN LONG-TAILED MACAQUES (*MACACA FASCICULARIS*) OF PENINSULAR MALAYSIA

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2017

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Leptospirosis and brucellosis are important zoonotic diseases worldwide with high incidence in tropical countries affecting biodiversity, human and livestock health, animal welfare and the economy (OIE, 2014; WHO, 2011). The expanding human population along with rapid urbanization have increased the likelihood of wildlife and human interaction. In Malaysia, the increased human-macaque conflicts (Hambali, 2012) have resulted in the concern about zoonotic disease transmission. This study was conducted to determine the seroprevalence of leptospirosis and brucellosis in wild long-tailed macaques of Peninsular Malaysia. A hundred serum samples were screened for antibodies against *Leptospira* and *Brucella* using microscopic agglutination test (MAT) and Rose Bengal Plate test (RBPT) respectively. Fourteen percent of macaques were seropositive for leptospirosis with serovar Cellodoni (4%), and Pyrogenes (4%) as the most common serovar identified, followed by Icterohaemorrhagiae (3%), Bataviae (2%) and Lai (1%).

The prevalence in males were significantly higher than females. Males were 4.5 times more likely to be seropositive for leptospirosis compared to females. This suggests that sex differences in behaviour influences exposure of macaques to leptospirosis. There were no significant difference in seroprevalence with age, habitat and region. All samples were seronegative for brucellosis. This study concludes that leptospirosis are prevalent in long-tailed macaques and poses a public health risk of cross-species transmission.

Keywords: *Leptospirosis, Brucellosis, Microscopic agglutination test (MAT), Rose Bengal Plate test (RBPT), Zoonosis, Long-tailed macaques (Macaca fascicularis), Non-human primate, Peninsular Malaysia*

1.0 INTRODUCTION

Zoonotic diseases are defined as diseases and infections which are naturally transmitted between vertebrate animals and man (WHO, 2017). The trend on wildlife emerging infectious diseases have been associated to the increase population density, encroachment into wildlife habitat, mismanagement of captive wildlife, change in agriculture practices, climate change, wildlife and exotic pet trade and ecotourism (Daszak et al., 2000; Guerra, 2013). Leptospirosis is an important worldwide zoonotic disease with high incidence in tropical countries, while brucellosis is a 'neglected zoonotic disease' (Thayaparan 2013; OIE 2014). Both diseases affects biodiversity, human and livestock health, animal welfare and economy (WHO 2011).

Leptospirosis is an endemic disease first reported in Malaysia in 1920 (Ministry of Health Malaysia, 2011) and has been recognized as a re-emerging public health problem in Malaysia (Arief, 2013). Factors for re-emergence are related to conditions favourable for maintenance and transmission of leptospirosis such as favourable reservoir and carrier hosts, flooding, animal-human interface and human host factors (Ministry of Health Malaysia, 2011). A seroprevalence study in Sarawak wildlife found 80% primates, 44% bats, 100% squirrels and 100% mongoose reacted positively to one or more serovars of *Leptospira sp.* (Thayaparan, 2013). Thayaparam (2013) emphasized on the importance of surveying wildlife species which lives at periphery of forests with potential to interact with humans, such as wild rats, carnivores and bats, but did not include non-human primates. Several seroprevalence studies conducted over the years revealed that non-human primates are susceptible to experimental leptospirosis and naturally acquired leptospirosis (Ibanez-Contreras et al 2010; Szonyi B 2011; Desvars 2013) and are clinically asymptomatic

(Astudillo et al. 2012). Although, not much is known on leptospirosis transmission between humans and non-human primates, non-human primates should be considered as a possible asymptomatic carrier (Szonyi, 2011).

Brucellosis is the most common zoonotic infection worldwide, but is particularly neglected in Asia, leading to the emergence of this disease (Pappas et al., 2006). This disease is endemic in Malaysia (Bamaiyi et al., 2014) and high number of cases have been reported among cattle populations under the integration-plantation system (Palanisamy et al., 1999). Livestock and wildlife interaction are drivers for disease transmission. Anka et al (2014) suggested that presence of wildlife and non-cattle species on same farm are significant to bovine brucellosis in Malaysia. Prevalence studies on animal brucellosis in Malaysia has been done on goats, cattle, buffaloes and dogs (Bamaiyi et al., 2014). The epidemiology and ecology of wildlife brucellosis is still poorly understood (Godfroid et al. 2013). Multiple studies have found that non-human primates are good models to study human brucellosis (Henning, 2011; Yingst, 2010). A novel Brucella sp. was isolated in wild caught baboons (Schlabritz-Loutsevitch et al, 2009), but no studies have been performed locally on seroprevalence of brucellosis in non-human primates.

The long-tailed macaques has dominated the human- wildlife conflict complaints received by the Department of Wildlife and National Parks in Malaysia (Saaban et al., 2016). The increase in human-macaque conflicts in Malaysia driven by loss of habitat and food sources, supported by subsequent adaptation to urbanized human environments result in higher interactions of humans and macaques (Hambali, 2012). Other contact opportunities such as feeding in public recreational areas, capture of wild macaques for the pet trade or

biomedical research colonies, consumption, or population management by wildlife authorities also increases human-macaque contact, thus increasing direct and indirect exposure to macaque body fluids (Lee et al., 2015). It is important to screen for particular species that lives at the periphery of forests and have the potential to interact with humans (Thayaparan et al., 2013), especially when wildlife serves as sinks for human pathogen (Muehlenbein, 2013).

No recent studies have been done to investigate seroprevalence of leptospirosis and brucellosis in primates of Peninsular Malaysia. The increase human-macaque conflicts and interactions in Malaysia which poses public health risk enhance the need to understanding prevalence of zoonotic diseases in the macaques.

This study was conducted to investigate the following objectives:-

1. To determine seroprevalence of leptospirosis and brucellosis in long-tailed macaques (*Macaca fascicularis*) of Peninsular Malaysia.
2. To investigate the association between seroprevalence of the two diseases to risk factors such as age, sex, habitat and region of long-tailed macaques (*Macaca fascicularis*).

The hypotheses of this study are:-

1. H_o: Long-tailed macaques of Peninsular Malaysia are seronegative for leptospirosis and/or brucellosis.
2. H_a: Long-tailed macaques of Peninsular Malaysia are seropositive for leptospirosis and/or brucellosis.

In this study, prevalence and risk factors for leptospirosis and brucellosis in long-tailed macaques are described using sera samples from human-macaque conflict cases reported in Peninsular Malaysia from 2015 to 2016. This study provides useful pilot information on the prevalence of these important zoonotic diseases in the macaques for more well designed studies in the future. The association between prevalence of disease and risk factors also gives us an insight on the possible effects of social structure and demography on disease persistence in primates.

2.0 LITERATURE REVIEW

2.1 Zoonotic threat of primates

In 1880, Rudolf Virchow introduced the word 'Zoonosis'. In 1959, the World Health Organization defined zoonotic diseases as 'those diseases and infections which are naturally transmitted between vertebrate animals and man' (WHO, 2017). A comprehensive review by Cleaveland et al., (2001) showed that 61% of 1415 human pathogens are zoonotic with 75% zoonotic emerging zoonotic pathogens (Cleaveland et al., 2001). Zoonotic diseases are thus important to both veterinary and human medicine, and public health (Burgos-Rodriguez, 2011). The trend on wildlife emerging infectious diseases have been associated to the increase population density, encroachment into wildlife habitat, mismanagement of captive wildlife, change in agriculture practices, climate change, wildlife and exotic pet trade and ecotourism (Daszak et al., 2000; Guerra, 2013).

Humans and nonhuman primates (NHP) share anatomical and physiological similarities, with NHP sharing approximately 25% of human emerging infectious diseases. These NHP can act as potential reservoir for zoonotic diseases. Humans are exposed to zoonotic pathogens from bites, scratches and accidental contact with body fluids (Burgos-Rodriguez, 2011).

2.2 Long-tailed macaques

The long-tailed macaques (*Macaca fascicularis*) are also known as the crab-eating macaque, cynomolgus monkey or 'kera. This species of primate is native to Southeast Asia (Cawthon-Lang, 2006) and can be found East and West Malaysia (Ong et al., 2008).

Macaques belong to the family of Cercopithecidae, the Old World Monkeys which represents the largest and most diverse primate families (Xing et al., 2005).

These primates have black skin on the feet and ears and generally covered with light brown fur with dark brown fur covering on their backs, legs and arms, and lighter undersides activity. Males weigh 3.5-8.3 kg, while females weigh 2.5-5.7 kg (Global Invasive Species Database, 2015). Long-tailed macaques can live up to 37 years old in captivity (Global Invasive Species Database, 2015). The age can classified into infant (<6 months old), juvenile (1-3 years old), subadult (3-6 years old) and adults (>6 years old) based on dental eruption (Seethamchai et al., 2008).

Long-tailed macaques live in troops of 12 to 25 (Fooden 1995). They are primarily arboreal animals that can leap five meters from one tree to another using their long tails for stabilization (Rodman, 1991). However, the crab-eating macaques can survive in a wide range of habitats including riverine, secondary and primary forest, forest periphery, mangrove and nipa swamp, coastal forest, and urban and agricultural settlings, in both natural and introduced range with preference for secondary habitats where habitats have been disturbed by human activity (Global Invasive Species Database, 2015).

2.3 Leptospirosis

Leptospirosis is a zoonotic disease caused by a spirochete from family Leptospiraceae, genus Letospira with ubiquitous distribution (Levett, 2001; Astudillo, 2012). *Leptospira sp*. are traditionally classified to *L. interrograns* which causes pathogenic leptospirosis and *L. biflexa* which is a saprophytic, free-living waterborne strain (Astudillo, 2012). There are

over 200 recognized serovars of *L. interrogans* and 60 serovars of *L. biflexa*. Serogroups are groups of serovars that are antigenetically similar which have no taxanomic standing, but useful for epidemiological understanding. There are 24 serogroups (Levett, 2001).

Leptospires are Gram negative, obligate aerobes that are tightly coiled spirochetes of 0.1µm by 6 to 0.1 by 20 µm with pointed ends usually bent into a distinctive hook (Levett, 2001). They possess translational and nontranslational forms of movement (Berg et al., 1978) and grow optimally at temperature of 28-30°C (Faine et al., 1999). Its survival in the environment is favoured by moisture, moderately warm temperature, and neutral or mildly stagnant water (Bolin, 2000).

2.3.1 Epidemiology

Leptospirosis is presumed to be the most widespread zoonosis globally (WHO, 1999) causing major economic impact on livestock industry (Ellis, 2015). It was recognized early as an occupational disease which later developed to a recreational disease and is significant in tropical agricultural environment and high seasonal rainfalls (Levett, 2001; Bharti et al., 2003; Guerra, 2013). It has a worldwide distribution with higher human infections in the tropics than temperate region due to increased survival of leptospires in warm and humid environment (Levett, 2004; Bharti et al., 2003).

Leptospires gain entry to human and animal body through abrasion or small cuts, via mucous membranes such as conjunctiva by inhalation of water or aerosol or through wet skin (Levett, 2001; Adler, et al., 2010). Most often, humans acquire the disease directly or indirectly from animal source as they rarely become chronic carriers (Adler, et al., 2010).

Infections in human are common in warm, moist climates with poor sanitation, poor rodent control and mixed domestic animal management system which provides the environment for survival of leptospires (Ellis, 2015).

Infected animals can become maintenance hosts or incidental hosts depending on the infective serovars after direct or indirect contact with urine or tissues of infected animals. Maintenance host excrete leptospires in its urine, contaminating soil, surface water, streams and rivers, while infection in incidental hosts causes severe or fatal disease with limited shedding time (Adler, 2010; Levett, 2001; Ellis, 2015). Example of host-serovar association that are of global importance are the *Rattus* species and serovar Icterohaemorrhagiae, cattle and serovar Hardjo, and sheep and serovar Canicola (Bharti et al., 2003; Ellis, 2015). Humans are dead-end in the chain of transmission (Minette, 1966).

Leptospirosis is an endemic disease first reported in Malaysia in 1920 (Ministry of Health Malaysia, 2011) and has been recognized as a re-emerging public health problem in Malaysia (Arief, 2013). Incidence of human Leptospirosis is highest in west-coast states of Peninsular Malaysia namely, Selangor, Perak, Kelantan and Pahang, with most cases reported during heavy rainfall and flooding (Benacer et al, 2016). Factors for re-emergence are related to conditions favourable for maintenance and transmission of Leptospirosis such as favourable reservoir and carrier hosts, flooding, animal-human interface and human host factors (Ministry of Health Malaysia, 2011)

Rodents are recognized as primary carrier, however other domestic animals like dogs, cattle, sheep and pigs also acts as maintenance host for several *Leptospira sp.* serovars.

Wildlife species in Malaysia where leptospires have been isolated are the palm civets, bats, porcupines, mouse deer and aquatic fish-eating snakes. A study in Sarawak showed seropositivity in monkeys, mongoose, squirrels and bats (Bahaman et al, 1988; Thayaparan, 2013), while a study on 36 primates in Selangor in 1961 showed no evidence of leptospirosis infection (Gordon-Smith et al, 1961). A study by Veterinary Research Institute in 2013 revealed that 35.10% buffalo, 30.16% horse, 27.26% cattle, 9.77% human, 2.37% sheep and 0.83% goat were seropositive for leptospirosis using MAT (Samsi et al., 2013).

2.3.2 Pathogenesis

One to two days after entry into the body through mucous membranes, skin abrasion and cuts or wet skin, bacteremia occurs lasting for a week. Production of circulating antibodies after 10-14 days ends the primary bacteremia stage. A secondary bacteremia stage is rarely reported. Young animals often develop acute clinical disease, thus causing haemolytic disease, hemoglobinuria, jaundice and death. Renal damage occurs in dogs and agalactia may occur in cattle, sheep and buffalo (Ellis, 2015). The motility of leptospires contribute to virulence of the organism enabling it to swim through viscous media leading to dissemination and end-organ damage to lungs, liver, kidney, eye and brain (Bharti et al., 2003).

Circulating antibodies produced are directed against serovar-specific leptospiral lipopolysaccharide which stimulates Toll-like receptor (TLR) 2 activating macrophages to remove Leptospires from circulation and tissue by opsonophagocytosis (Bharti et al., 2003; Adler, 2010). Circulating antibodies reach maximum levels at week 3 – 6 post-infection

and maintain for up to 6 weeks depending on species, followed by gradual decline. Low titres are detectable in animals for many years (Ellis, 2015). Antibodies may fall to undetectable levels while animals remain chronically infected (OIE, 2008).

Recovered animals may develop into asymptomatic carriers harbouring virulent leptospires in proximal renal tubules for long periods and shedding leptospires into the environment (Levett, 2001; Adler, et al., 2010). Duration and intensity of shedding of multiplying leptospires in proximal renal tubules are dependent on species, individual animals and infecting serovar. Leptospires may localize in uterus of pregnant animals transmitting disease via intrauterine and aborted materials (Ellis, 2015).

2.3.3 Clinical manifestation

Humans and domestic animals, especially dogs, cattle and swine succumbs to the systemic infection thus causing fever, renal and hepatic insufficiency, pulmonary disease and reproductive failure. Clinical signs are variable depending on infective serovars (Adler et al., 2010). Leptospirosis affecting livestock causes major economic loss due to abortions, stillbirths, birth of weak neonates, death, reduce milk production, cost of veterinary care, treatment and vaccine and decline of export (Bolin, 2000).

2.3.4 Screening and diagnosis

The diagnosis of leptospirosis in animals are important for confirming clinical diagnosis, determine herd prevalence, in epidemiological studies and to assess infectivity status for international trade or introduction into an uninfected herd (OIE, 2008; Ellis, 2015).

Laboratory diagnosis of leptospirosis are made by demonstration of antigen or by demonstration of antibodies against leptospirosis (Adler et al., 1990; OIE, 2008). The demonstration or isolation of leptospires in internal organs and body fluids of symptomatic animals provides a definitive diagnosis of an acute clinical disease, whereas presence in the kidney, urine, or genital tract of asymptomatic animals is diagnostic only of chronic carrier state (OIE, 2008), as leptospires localize in the immunologically protected sites (Ellis, 2015). The methods used to demonstrate antigens are dark field microscopy, culture, DNA detection methods and staining (Ellis, 2015).

Dark field or phase-contrast microscopy of wet mount is used for direct visualization due to poor staining characteristic of bacteria. The dark-field microscope was designed to focus light on the specimen in an oblique angle, therefore light passing through the slide do not enter objective lens, enabling leptospires to appear bright on a dark background. (Feto et al., 2008). However, this technique is not recommended due to high number of false-positivity and false-negativity (Bharti et al., 2003).

Isolation of leptospires is performed by culturing fresh samples in semisolid medium with addition of selective agents to prevent growth of other bacteria, incubated for 16-26 weeks and examined using dark-field microscopy every one to two weeks (OIE, 2008). Culture

lack sensitivity, requires specialized culture media and long duration of culture, and thus is not useful as routine test for diagnosis (Adler et al., 2010; Bharti et al., 2003).

Molecular methods using polymerase chain reaction (PCR)-based assay are used to detect leptospires DNA in samples. It utilizes primers for specific genus of *Leptospira* or designed to only identify pathogenic species. Unfortunately, PCR primers for testing animal samples are undeveloped and test lacks specificity due to presence of amplification inhibitors in samples (OIE, 2008).

The two most useful serological test in veterinary diagnosis are the microscopic agglutination test (MAT) and enzyme-linked immunosorbent assay (ELISA). Microscopic agglutination test (MAT) is the most widely used diagnostic test (Ellis, 2015; Adler et al., 2010) and is a reference standard test or gold standard for serological diagnosis due to its high sensitivity and unsurpassed diagnostic specificity (Goris et al., 2014; Adler et al., 2010). It is useful for diagnosing acute infection using paired serum samples and for identifying herd status, however, chronically infected animals that aborted or are shedding may have titres below the widely accepted titre of 1/100 (OIE, 2008). The reactive serum suggests but do not identify the infecting serovar due to cross reactivity of serovars (OIE, 2008) due to the antibody produced against leptospiral LPS that are restricted to antigenically related serovars (Adler, et al. 2010). One serovar may belong to many species (Bharti et al, 2003). MAT do not distinguish between antibodies from infection or vaccination (Adler, et al. 2010). Some of the sensitivity and specificity of MAT reported in human studies are 65.6% clinical sensitivity, 97.7% specificity, 54.9% subclinical sensitivity and 97.3% subclinical specificity (Schlichting et al., 2015), 77.4% sensitivity

and 97.6% specificity (Niloofa et al., 2015). Results reported in veterinary studies are 59.11% sensitivity and 96.45% specificity in canine (Kumar et al, 2013), 100% sensitivity and 100% specificity for California Sea Lions (Colagross-Schouten, 2002),

The enzyme-linked immunosorbent assay (ELISA) to detect antibodies against leptospires have been developed using different antigen, assay protocols and assay platforms (OIE, 2008). ELISA is not useful in identifying the most probable infecting serovar or serogroup, but can detect different class of antibody (Mohammed, 2011). IgM can be detected a week after infection or IgG 2 weeks after an infection in humans and animals (OIE, 2008; Budihal, 2014). Kumar (2013) recommend that indirect ELISA is valuable for routine laboratory diagnosis due to its high sensitivity, rapid and easy to perform procedure. The antigenic preparation of ELISA in large quantities and long storage period also excludes the need to maintain a constant supply of live leptospiral cultures for different serovars compared to MAT (Kumar, 2013). The use of ELISA alone for diagnosis is not recommended as its sensitivity and specificity is low compared to MAT (Adler, et al. 2010).

2.4 Leptospirosis in non-human primates

A serological survey by Minette in 1966 on 1420 sera representing 34 species of primates showed that leptospirosis is uncommon in non-human primates (7% seroprevalence). Old World Monkey have higher infection rate compared to New World primates which could be due to the arboreal nature of New World primates which reduces contact with contaminated soil or affected ground rats (Minette, 1966; Lilenbaum et al., 2005). Naturally acquired infections have been reported at 4.8% in bonnet monkeys from the

forest in India and 3.7% in free-living urban rhesus monkeys in India (Minette, 1966). Serogroup Icterohaemorrhagiae was most commonly found in Minette's study (1966) with other positive serogroups of Ballum, Grippotyphosa, Australis, Bataviae, Pyrogenes and Sejroe.

Although studies by Minette (1966) claimed that anti-leptospiral antibodies do not persist for long periods of time in some primate species or are not developed in certain serotypes, an experiment by Everard (1991) showed that naturally acquired leptospiral agglutinins can persist for five years or more in some monkeys, while some lasted within a year. Naturally-acquired anti-leptospiral antibodies in Vervet monkeys can last more than two and a half years (Baulu, 1987)

A serological survey in Netherlands performed on 104 captive non-human primates resulted in 16% seropositive subclinical individuals using MAT with titers of 80 or more. The reactive serovars were Bratislava, Icterohaemorrhagiae, Copenhageni, Poi and Pomona (Jaffe et al., 2007). Vervet monkeys in Barbados were 29.9% seropositive at titers of $\geq$1:100 and 5.4% seropositive at titers of 1:50 (Baulu, 1987). Captive primates in Salvador, Brazil were 56.8% seropositive for leptospirosis with Icterohaemorrhagiae as the most frequent serogroup, followed by serogroup Canicola (Pinna et al., 2012).

A serological study of Leptospiral infection in wildlife in Sarawak showed that four out of five monkeys which includes two captive primates (*Macaca nemestrina, Hylobates muelleri*) and two free-ranging primates (*Presbytis cristata, Nasalis larvatus*) were

seropositive for serovar Lepto 175 Sarawak and serovar Lai. One captive primate (*Macaca fascicularis*) was seronegative (Thayaparan et al., 2013).

Multiple literatures have reported that primates experience subclinical infection of leptospirosis (Lilenbaum, 2005; Baulu, 1987; Astudillo et al, 2012, Pinna et al., 2012), some with mild illnesses such as fever and weight loss (Minette et al., 1968). Some species like Squirrel monkeys (*Saimiri sciureus*) may experience clinically acute leptospirosis from *L. icterohaemorrhagiae* infection that are sometimes fatal, with signs of icterus and minimal hepatic lesions accompanied with markedly increased indirect bilirubin and normal transaminase levels (Minette et al., 1968). A severe leptospirosis outbreak was reported in captive Capuchin monkeys with clinical signs of icterus, lymphadenopathy, and with 27% mortality due to unhygienic and improper husbandry (Szonyi et al., 2011).

The transmission of Leptospirosis between humans and non-human primate is unknown (Szonyi et al., 2011)

2.5 Brucellosis

Brucellosis was discovered by Sir David Bruce in 1887 in a soldier in Malta (Godfroid et al., 2013). It is now a 'neglected zoonotic disease', which are zoonotic diseases that mainly affects poor and marginalized populations in low-resource settings especially in Asia (MyIrea, 2015). Other names for brucellosis are Bang's disease, Brucellemia, Cyprus fever, Malta fever, Rock fever, Typhomalarial fever and Undulant fever (Berger, 2016).

Brucella sp. is a member of the Brucellaceae family, in order Rhizobiales, class Alphaproteobacteria (OIE, 2016). It is a Gram negative, cocco-bacilli, non-spore forming, non-capsulated facultative intracellular bacteria (Seleem et al., 2010). Ten species have been recognized and are divided into groups of 'classical' Brucella species (*B. abortus* in cattle*, B. melitensis* in sheep and goats*, B. suis* in pigs*, B. ovis* in sheep*, B. canis* in dogs*, B. neotomae* in wood desert rats) and 'new' Brucella species (*B. ceti, B. pinnipedialis, B. microti, B. inopinata*) due to microbiological, molecular, host preference and pathogenicity differences (Godfroid et al., 2013; Figueiredo, et al., 2015). *B. abortus, B. melitensis* and *B. suis* are highly pathogenic in humans (OIE, 2016). While, *B. canis* has a mild zoonotic potential to humans (Xavier, 2010; Alton et al., 1996).

Host preference of the organism exists, but *B. abortus* and *B. suis* have been isolated in wildlife species. *B. melitensis* is rarely reported in wildlife (Godfroid et al., 2013). Reports have shown that the organisms may infect other animals other than their primary host causing a self-limiting infection (Ficht, 2010).

2.5.1 Epidemiology

Brucellosis is a widely distributed zoonotic bacterial disease in mammals (Godfroid et al., 2013). There are more than 500,000 human cases reported annually (Seleem, et al. (2009). The global disease burden on livestock is massive at estimates of >300 million affected out of the 1.4 billion worldwide cattle population (Figueiredo, et al., 2015). The occurrence of the disease depends highly on infected animal reservoirs (Godfroid et al., 2013). These animal reservoirs can range from wild, feral and domestic animals (Alton et al., 1996).

Brucellosis is an occupational health hazards to abattoir, animal industry, hunters and health workers (Godfroid et al., 2013).

Humans are infected through ingesting contaminated animal food products, such as, raw milk especially in developing countries (Godfroid et al., 2013), through direct contact with infect animals or by inhalation of aerosol droplets by accident or as result of bioterrorisms (Purcell et al., 2007). An outbreak in Pulau Pinang, Malaysia occurred in 2012 involved 79 individuals due to ingestion of unpasteurized goat milk (Leong, et al., 2015). This disease rarely transmits from person to person (Purcell et al., 2007).

A study by Anka et al. (2013) based on 8-years of serological data in Malaysia from year 2000-2008 showed that *B. abortus* antibodies were detected in 21.8% of sampled bovine herds and was increasing and 2.5% of sampled cattle. Brucellosis in livestock of Malaysia has been associated with importation of unknown disease status breeder herds, and are high in cattle reared under the integration farming system (Zamri-Saad et al., 2016). Other common risk factors in animals include high stocking density, large herd sizes of farms, mixed farming and inadequate biosecurity (Bamaiyi, 2016).

2.5.2 Pathogenesis

Upon entering the body through mouth, conjunctivae, respiratory tract and abraded skin (Alton et al., 1996), the bacteria is taken up by local tissue lymphocytes, and transferred through regional lymph nodes into the circulation. Once in the circulation, the brucellae will be seeded throughout the entire body with strong tissue tropism for lymphoreticular

and reproductive systems (Figueiredo, et al., 2015; Pappas et al., 2005). Incubation period in humans is 2-3 weeks (Corbel, 2006).

Its ability to infect intracellularly enables avoidance to the innate and adaptive immune response and effects of antibiotics (Figueiredo, et al., 2015). Tissue lesions in humans caused by brucellosis are in forms of minute granulomas composing of epitheloid cells, polymorphonuclear leukocytes, lymphocytes and some giant cells. Infection by *B. suis* may cause abscesses (Alton et al., 1996). When septic abortion in animals occurs, high concentration of bacteria is shed during parturition and aerosolized in body fluids, thus infecting other animals and humans (Purcell et al., 2007).

Pathogenesis in wildlife reservoirs are not yet fully defined (Godfroid et al., 2013).

2.5.3 Clinical manifestation

Brucellosis in humans are manifested as an acute febrile illness which can persist and progress into a chronic disease accompanied by severe complications such as, osteoarticular diseases (Corbel, 2006; Pappas et al., 2005). Brucellosis signs include intermittent fever or remittent fever, malaise, anorexia and prostration. Chronically infected humans will develop persistent localized infection or non-specific syndrome resembling the 'chronic fatigue syndrome' (Corbel, 2006). Lymphadenopathy, hepatomegaly or splenomegaly may be present (Pappas et al., 2005). *B. melitensis* is most pathogenic to humans, whereas *B. abortus* are more commonly manifested with sub-clinical cases (Alton et al., 1996).

Brucellosis in animals are manifested with one or more of the following signs such as, abortion, retained placenta, placentitis, orchitis, epididymitis and rarely arthritis (OIE, 2016; Poester et al., 2010). Abortion is the most important clinical sign and occurs at the first gestation. Females usually abort once, but remain infected for life (Godfroid et al., 2010).

2.5.4 Screening and diagnosis

Laboratory testing are important in diagnosis of brucellosis due to its non-pathognomonic signs (Godfroid et al., 2013). Presumptive diagnosis of brucellosis can be done through demonstration of Brucella-like organisms in aborted materials or vaginal discharge using modified acid-fast staining (Godfroid et al., 2013). However, *Chlamydophila abortus, Chlamydia psittaci* and *Coxiella burnetti* are morphologically similar to Brucella sp. which can mislead diagnosis (Kaltungo et al., 2014).

Definitive diagnosis of brucellosis is by isolation of *Brucella spp.* or specific DNA detection by PCR using classical or molecular techniques to identify and type specific strains (Godfroid et al., 2013). Isolation of *Brucella spp*. can be done by culturing samples from uterine discharges, aborted foetuses, udder secretions, lymph nodes or reproductive organs (OIE, 2016). Blood cultures are only useful in individuals with bacteremia (Kaltungo et al., 2014).

Serological tests such for screening of herds are available, such as, Rose Bengal plate (RBPT) and buffered plate agglutination test, complement fixation test, enzyme-linked immunosorbent assays (ELISA) or fluorescence polarisation assay. RBPT is recognized as

a quick, cheap and effective test for diagnosis of brucellosis as it only requires simple equipment and results can be read using the naked eye (MacMillan, 1997). The high sensitivity of RBPT could sometimes lead to false positive results for antibodies due to vaccination and thus, require subsequent confirmatory procedures (OIE, 2016). It is also highly sensitive in acute and chronic brucellosis due to its ability to detect IgM, IgG and IgA, to absence of prozones and to agglutinating activity of blocking IgA (Diaz et al., 2011). Cross-reactions may occur with other bacteria such as, *Francisella tularensis, Escherichia coli O116* and *O157, Salmonella urbana, Yersinia enterocolitica O:9, Vibrio cholerae, Xanthomonas maltophilia,* and *Afipia clevelandensis* (Pappas et al., 2005). Sensitivity and specificity for RBPT were reported to be 92% and 94% (Salman et al., 2012). RBPT is recommended by OIE (2016) as a general purpose diagnostic test in all wildlife species.

Complement fixation test is the recommended gold standard for serological diagnosis of brucellosis CFT acts as a confirmatory test for positive RBPT samples (Yusof et al., 2012). Indirect ELISA or milk ring test are often performed on bulk milk samples as effective screening and monitoring in dairy herds (OIE, 2016). Milk ring test (MRT) detects IgM and IgA but only has 72% sensitivity and 80% specificity (Kaltungo et al., 2014; AlMariri et al., 2010). I-ELISA and C-ELISA seems useful for serological surveys in wildlife, however lack of validation studies make interpretation difficult (OIE, 2016). Brucellin skin test is done for screening or confirmatory test for positive serological reactors in absence of risk factors (OIE, 2016). It is highly specific which is good for herd test, but its low sensitivity lacks in individual diagnosis (Godfroid, 2010).

2.6 Brucellosis in non-human primates

Literature on non-human primate brucellosis have been minimum with a case of *B. melitensis* reported in *Papio spp*. by Pinkerton (1972). In 2009, a novel Brucella isolate was discovered from a wild-caught 13-year-old baboon in Tanzania and an 8-year-old colony-born baboon. Both baboon had stillbirth and retained placenta (Schlabritz-Loutsevitch et al., 2009). The proposed name for this new *Brucella* species is *Brucella papionis sp. nov*. with the type strain F8/08-60^{T} (Whatmore, et al., 2014). The recent finding of a novel *Brucella sp.* isolate posed new challenges to our understanding of brucellosis at the wildlife/livestock/human interface (Godfroid et al., 2013).

Experimental brucellosis have been successful in infecting macaques (stumped-tail macaques and rhesus macaques) through oral, conjunctival, intravenous and aerosol route of brucellosis with *B. melitensis, B. suis, B.cabortus* and *B. canis* (Yingst et al., 2010; Henning, et al., 2011; Percy et al., 1972; Mense et al., 2004). Infected macaques had waxing and waning fever, lymphadenopathy as early as 3 days post-inoculation (Yingst et al., 2010), while some studies reported asymptomatic infection up to five weeks post-inoculation (Percy et al., 1972). Pathology showed splenomegaly, hepatitis, orchitis, epididymitis and lymphadenopathy with focal granulomatous lesions in the affected organs which are similar to those described in humans (Percy et al., 1972; Mense et al., 2004). Experimental studies have shown that some non-human primates produce antibodies against *B. abortus* and *B. melitensis* (Wilson, 1936).

2.7 Human –Macaques conflict

The International Union for the Conservation of Nature (IUCN) World Parks Congress in 2003 defined human-wildlife conflict (HWC) as occurring 'when wildlife requirements encroach on those of human populations, with costs both to residents and wild animals' (IUCN, 2005). World Wildlife Fund (WWF) defined that HWC are 'any interaction between humans and wildlife that results in negative impacts on human social, economic or cultural life, on the conservation of wildlife populations, or on the environment' (WWF, 2005).

The two macaque species listed in the Global Invasive Species Database (2015) are the long-tailed macaques (*M. fascicularis)* and the rhesus macaques (*M. mulatta*). Invasive species are species that are non-native to the ecosystem in which introduction is likely to cause harm to the economy, environment and human health (The National Invasive Species Council, 2006). Wildlife populations serve as sink for human pathogens (Muehlenbein, 2013).

A study from year 2006-2015 on the human-wildlife conflict complaints received by the Department of Wildlife and National Parks in Malaysia showed that long-tailed macaques (66%, which is 56,786 complaints) was most reported, followed by elephants (9%), wild boar (7%), common palm civet (6%), pig-tailed macaque (3%), and others (DWNP, 2016). The total number of long-tailed macaques as reported by DWNP in 2011 is 133403 individuals with 5% population growth rate per annum. Agricultural areas recorded highest population estimate (Karuppannan et al., 2014).

The increase in human-macaque conflicts in Malaysia is driven by loss of habitat and food sources. Subsequent adaptation to urbanized human environments result in higher interactions between humans and macaques (Hambali, 2012). Other contact opportunities such as feeding in public recreational areas, capture of wild macaques for the pet trade or biomedical research colonies, consumption, or population management by wildlife authorities also increases human-macaque contact, thus increasing direct and indirect exposure to macaque body fluids (Lee et al., 2015).

Reports of conflicts have been made such as, attacks, snatching, bites, stealing and urinating and defecating in homes, (Murali, 2017; FMT, 2015; BBC, 2010; Hambali et al., 2012, Md-Zain et al., 2014). Multiple reports of attacks and bites have also been reported by individuals who keep monkeys as pets (FMT, 2016).

3.0 MATERIALS AND METHODS

3.1 Study design and source of samples

Serum samples from long-tailed macaques have been routinely collected from captured macaques in conflict areas and stored at -80˚C at the Veterinary Parasitology Laboratory, Faculty of Veterinary Medicine of Universiti Putra Malaysia. The macaques were captured and translocated throughout Peninsular Malaysia by the Department of Wildlife and National Parks (DWNP/PERHILITAN) for the macaque management program. From this collection of samples, 100 serum samples were selected. Information available pertaining to the samples selected such as, age, sex, type of habitat, location and GPS coordinates were extracted (Table 1).. All macaques appeared clinically healthy during sample collection.

3.2 Study area

The location of the macaque samplings divided into three regions, northern, middle and southern of Peninsular Malaysia.

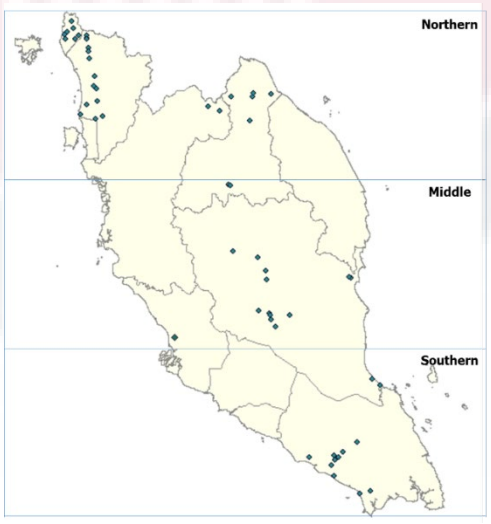

Figure 1: Mapping of macaque sampling in Peninsular Malaysia from macaques conflict areas

3.3 Determination of sample size

Sample size (n) for this study was determined using the Open-Epi Version 3 calculator. The software program applies the formula of, n = [DEFF*Np (1-p)]/[($d^2/Z^2_{1-\alpha/2}$ *(N-1)+p*(1-p)], where N = population size: 133403, p = prevalence = 50%, d = confidence limits = 10%, and DEFF = design effect for cluster surveys = 1. The estimated sample size used in the current study with the confidence levels of 95% was n = 96.

3.4 Data analysis

Descriptive analysis were performed and seroprevalence were calculated as the percentage of those seropositive over those tested. The association between several factors such as sex and age of wild long-tailed macaques and seroprevalence of leptospirosis and brucellosis were analysed using Pearson Chi-Square test at significance level $\alpha = 0.05$.

3.5 Serology: Leptospirosis

Microscopic agglutination test (MAT) was performed according to the protocol described by WHO in the OIE Terrestrial Manual 2008 for leptospirosis. Antigen live cultures of 12 serovars of Leptospira (Cellodoni, Cynopteri, Canicola, Hebdomadis, Icterohaemorrhagiae, Pomona, Malaysia, Lai, Hardjo bovis, Grippotyphosa, Bataviae, Pyrogenes), representing 11 different serogroups were used. These antigen live cultures have been cultivated in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium which contains 1% bovine serum albumin and Tween 80 (source of long-chain fatty acids) for 7-10 days at 30°C.

A two-fold serial dilution of serum was made starting at 1:25 to 1:25600 in phosphate buffer solution (PBS) in microtiter plates. Then, antigen live culture was mixed in all wells. After incubation at room temperature for 12-24 hours or 2 hours at 37°C, results were read using a dark-field microscope. The end-point titer was determined as the highest serum dilution which ≥50% of leptospires have remained agglutinated (Faine et al., 1999). Serum was considered positive if titer was ≥1:50 in the MAT.

Table 2. Twelve *Leptospira* serovars used in the study for MAT with its strain, serogroup and species

No.	Serovar	Strain	Serogroup	Species
1	**Bataviae**	Swart	Bataviae	*L. santarosai*
2	**Cellodoni**	Celledoni	Cellodoni	*L. weilii*
3	**Cynopteri**	3522C	Cynopteri	*L. kirschneri*
4	**Canicola**	Hond Utrecht IV	Canicola	*L. interrogans*
5	**Grippotyphosa**	Moskva V	Grippotyphosa	*L. kirschneri*
6	**Hardjo bovis**	117123	Sejroe	*L. borgpetersenii*
7	**Hebdomadis**	Hebdomadis	Hebdomadis	*L. kirschneri*
8	**Icterohaemorrhagiae**	RGA	Icterohaemorrhagiae	*L. interrogans*
9	**Lai**	Lai	Icterohaemorrhagiae	*L. interrogans*
10	**Malaysia**	Bejo-Iso9T	Tarassovi	*L. kirschneri*
11	**Pomona**	Pomona	Pomona	*L. interrogans, L. noguchii*
12	**Pyrogenes**	Salinem	Pyrogenes	*L. interrogans*

Source: Levett, 2001

3.6 Serology: Brucellosis

Rose Bengal Plate test (RBPT) was performed according to the protocol described by WHO in the OIE Terrestrial Manual 2016 for brucellosis *(B. abortus, B. melitensis, B. suis).* The test was done manually with standardised *B. abortus* and *B. melitensis* antigens by the brucellosis Reference Laboratory in New Haw Addlestone, Surrey KT15 3NB, UK. Macaque serum and antigens were thawed to room temperature. Twenty five (25) µl of each serum samples and a drop of antigen were placed on a plastic plate. The plate was rocked and results was read within 3-4 minutes of mixture. Presence of agglutination were read as positive.

4.0 RESULTS

A total of 100 long-tailed macaque (*M. fascicularis*) serum samples were examined for leptospirosis and brucellosis. At time of sampling, all macaques appeared to be clinically healthy.

4.1 Leptospirosis

At cut-off positive titer of 1:50, 14% (14/100) were positive for MAT. The positive titers ranged from 1:50 to 1:200. Nine % (9/100) were seropositive at titers of ≥1:100, and 5% (5/100) were seropositive at titers of ≥1:200. No animals presented titers >1:200. Using the cut off titer of 1:50, the seroprevalence of leptospirosis is 14% (95% CI: 8.14-22.71).

The most common serovar identified were serovar Cellodoni (4%), and serovar Pyrogenes (4%), followed by serovar Icterohaemorrhagiae (3%), serovar Bataviae (2%) and serovar Lai (1%) (Figure 1). Most samples, 98/100 reacted to only one serovar. Two sera samples reacted to more than one serovar, one which reacted to two serovars (Bataviae and Cellodoni) and another which reacted to six serovars (Bataviae, Cellodoni, Grippotyphosa, Hardjo bovis, Icterohaemorrhagiae, and Pomona).

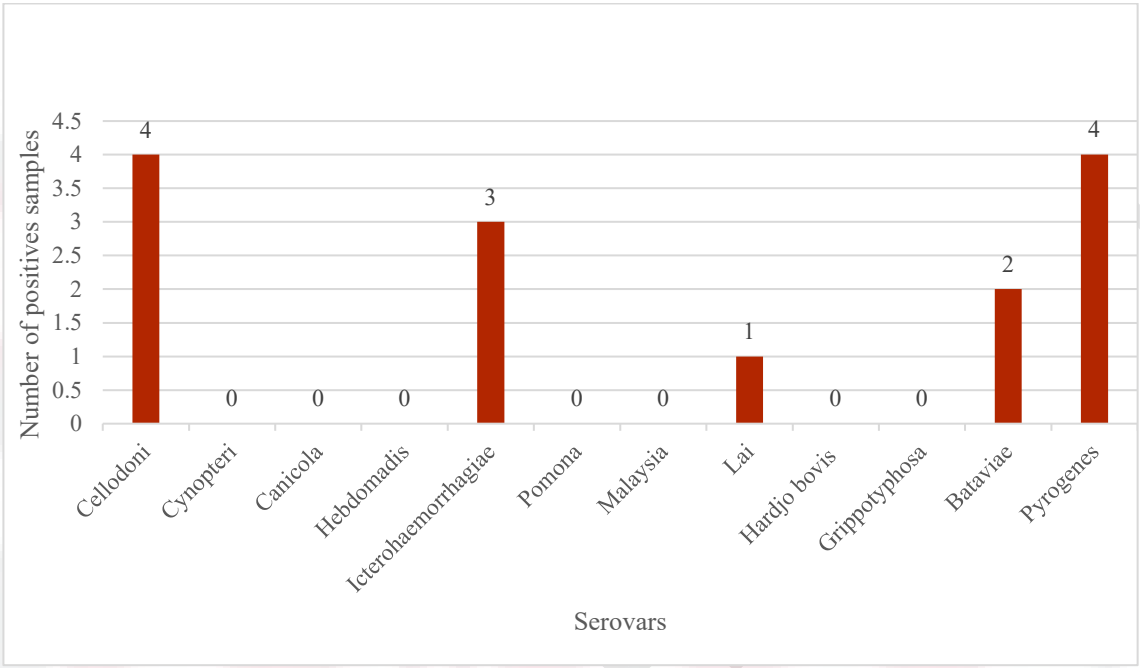

Figure 2**:** Infecting *Leptospira* serovars identified in long-tailed macaques from conflict areas in Peninsular Malaysia.

Seroprevalence was significantly higher (P<0.05) in males (12/61) as compared to females (2/39). Males were 4.5 times (Odds ratio 95% CI: 1.05-31.04) more likely to be seropositive for leptospirosis compared to females (Figure 2a). There were no significant difference in seroprevalence with risk factors of age (adult and sub-adult) (Figure 2b), habitat (secondary forest, plantation, sub-urban and urban) (Figure 2c) and region (northern, middle, southern) (Figure 2d). None of the macaques from the urban habitat were seroprositive for Leptospirosis.

Nine of 12 *Leptospira* serovars were detected in this study (Bataviae, Cellodoni, Grippotyphosa, Hardjo bovis, Icterohaemorrhagiae, Malaysia, Pomona, and Pyrogenes) at different titers (Table 3). No agglutination were detected with Cynopteri, Canicola, and Hebdomadis.

Table 3: *Leptospira* serovars detected in this study

No.	Serovar	Titers			
		1:25	**1:50**	**1:100**	**1:200**
1	Bataviae	6	4	2	0
2	Cellodoni	5	4	2	0
3	Cynopteri	0	0	0	0
4	Canicola	0	0	0	0
5	Grippotyphosa	1	1	0	0
6	Hardjo bovis	6	1	0	0
7	Hebdomadis	0	0	0	0
8	Icterohaemorrhagiae	7	4	2	2
9	Lai	*2*	1	1	0
10	Malaysia	1	0	0	0
11	Pomona	2	1	0	0
12	Pyrogenes	5	4	4	3
	Total	35	20	11	5

4.2 Brucellosis

All samples were seronegative for brucellosis by RBPT.

5.0 DISCUSSION

5.1 Leptospirosis

This study uses cut-off value of 1:50 which is lower than the cut-off value of 1:100 recommended by the OIE. Currently there is no established cut-off titer for this species or other non-human primate in this geographical area. We deduced that the cut-off titer for infected individuals of this species would be low because of several reasons; 1. endemicity of leptospirosis in Malaysia (Shivkumar, 2006) that creates constant low level exposure to most mammals 2. Many literatures reported asymptomatic infections in primates (Lilenbaum, 2005; Baulu, 1987; Astudillo et al, 2012, Pinna et al., 2012) 3. consideration that antibody titer in animals will decrease over time below the widely accepted minimum significant titer of 1:100 (OIE, 2008) and 4. limitation of MAT in interpreting low titer in a single serum (WHO, 2003). We believe that the use of a lower cut-off titer of 1:50 in this study reduces an underestimation of prevalence in the studied population.

The reports on leptospirosis in non-human primates in this region is scanty. The first and only prevalence study of leptospirosis in non-human primates in Peninsular Malaysia was performed in 1961. This study reported negative serological and negative culture findings for 16 wild long-tailed macaques (*M. fascicularis*), 15 Sunda slow loris (*Nycticebus coucang*) and four other species of primates (Smith et al., 1961). The 14% seroprevalence (14/100) in the present study shows that long-tailed macaques have been exposed to Leptospirae. This prevalence is less than studies conducted in Sarawak which revealed 80% seroprevalence (4/5) in different wild primate species, with absence of titer in the long-tailed macaque (Thayaparan et al., 2013). Another study in Sarawak revealed 75% seroprevalence (6/8) in captive primates and 50% seroprevalence (2/4) in wild primates

(Thayaparan et al., 2014). Sabah reported 88% seroprevalence (30/34) in wild *Pongo pymaeus* (Kilbourn et al., 2003). However, the sample size used in the aforementioned studies were small and therefore may not represent true disease situation in the population. On the other hand, the result of our study may be an underestimation of the prevalence, as MAT is not as sensitive as IgM ELISA in detecting acute and early convalescent stages of infection (Goris et al., 2012). In addition, antibody titer would decrease below the detectable level in chronic infection (OIE, 2008). Furthermore, lack of representative serovars in the test panel would also reduce sensitivity of MAT (OIE, 2008). The only local serovar used in this study was serovar Malaysia strain Bejo-Iso9T. However, our finding is consistent with the report from our neighbouring country which reported 10% seroprevalence (3/30) in wild-caught long-tailed macaques of Northeastern Thailand (Pumipuntu, 2015).

MAT is a reference standard test or gold standard for serological diagnosis of leptospirosis due to its high sensitivity and diagnostic specificity (Goris et al., 2014; Adler et al., 2010). The reactive serum in this study indicates the presumptive but not definitive infecting serovar or seroroup due to cross reactivity of serovars (OIE, 2008). Cellodoni (4%) and Pyrogenes (4%) were the most common infecting serovar identified in our study. These serovars are second (23.21%) and third (12.5%) predominant serovars in humans locally, with Cynopteri being the first (24.11%) (Samsi et al, 2013). Pyrogenes was reported in primates of Barbados (Baulu et al., 1987) and Brazil (Pinna et al, 2011), cattle (Leong et al., 1975, Joseph, 1979), pigs (Joseph, 1979) and dogs (Smith et al., 1961) in Malaysia. In a comprehensive study by Smith et al. (1961) of wildlife in Malaysia, serovar Pyrogenes, Icterohaemorrhagiae and Bataviae were found in house and forest ground rats. Tree shrews

were also positive for Pyrogenes. There are little studies which includes Cellodoni in the MAT panel for test. Cellodoni was found in 3.40% in 3430 samples of domestic animals and humans in Malaysia (Samsi et al, 2013), and 1.6% (22/558) in cattle, 0.9% (6/657) in goat, and 0.1% (1/869) in pigs of West Malaysia (Bahaman et al., 1987).

The finding of serovar Icterohaemorrhagiae is similar to other seroprevalence studies in wild non-human primates of Thailand (Pumipuntu, 2015), in captive non-human primates of Brazil (Pinna et al., 2011; Lilenbaum et al, 2002; Lilenbaum et al, 2005) and in reports of outbreak (Szonyi et al., 2011). This is not an unusual finding as Icterohaemorrhagiae have been reported as the most common serogroup infecting primates by Minette since 1966 from reviewing nine studies (Minette, 1966). Icterohaemorrhagiae is potentially one of the most pathogenic serovar to man (Minette, 1968) and is primarily isolated in the South East Asia region (Benacer et al, 2016; Victoriano et al, 2009). This serovar have been found in cattle, pig, goat, dog in Malaysia (Joseph, 1979) however its link to human infection have yet to be established.

Serovar Bataviae is found in cattle, pigs, dogs in Malaysia (Joseph, 1979), primates in Brazil (Pinna et al, 2011), and was the predominant serovar detected in dogs in Malaysia (Samsi et al, 2013). Serovar Lai is common in rats of Southeast Asia (Levett, 2001). Thayaparan (2013, 2014) reported serovar Lepto *175* (a new strain of an unknown serogroup) as the most common serovar among the non-human primate in Sarawak, followed by Lai, Pomona, Pyrogenes and Copenhageni. This study did not find any sera that reacted to serovar Hebdomadis which is the most frequent serovar in domestic animals

and humans in Malaysia (22.60%) (Samsi et al., 2013). The discovery of serovars Cellodoni and Bataviae in this study is the first in non-human primates of Malaysia.

Sex is a significant risk factor of leptospirosis in the long-tailed macaques with males being 4.5 times more likely to be infected than females. Higher prevalence of leptospirosis in males have also been reported in other species such as dogs (Ward et al., 2002) and humans, where both species are related to exposure bias (Haake, et al., 2015). We believe that the higher prevalence in male long-tailed macaques is due to emigration behavior of males as compared to females. Throughout his lifetime, male tends to move from one group to another to acquire a dominant rank as compared to females that remain in their natal groups. The average duration of residence of a in a group is 45 months before it migrates to another group (van Noordwijk et al., 1999; van Noordwijk et al., 2001). The act of emigration from one place to another increases the risk of exposure to infected animals and *Leptospira sp*. from the environment. Male long-tailed macaques also have significantly higher practice in ano-genital inspection of females manually, orally or olfactory (Karimullah, 2011). This behaviour increases the risk of male macaques to contract *Leptospira sp.* from these routes.

Age did not significantly affect prevalence of leptospirosis in the long-tailed macaques in our study. However, in this study we only have two age groups which are subadult and adult therefore is not representative of all age groups. We believe that there is little difference between the behaviour and nature of this two age group that would result in one to be exposed more so than the other.

Habitat and region were not significant in this study which could be attributed to the rather even distribution and endemicity of leptospirosis in Malaysia (Benacer et al, 2016). The climate condition which is warm with average temperature of 27˚C and wet throughout the year with annual rainfall exceeding 2000 mm (Benacer et al., 2016) allows for the persistence of the organism and the abundance of rats as the principle host of *Leptospira sp.* and other reservoir hosts provide a conducive environment for *Leptospira sp.* survival and spread (Levett, 2015; Bahaman et al., 1998; Bahaman et al., 1991; Levett, 2011). Leptospirosis cases in humans in the west coast of Peninsular Malaysia does not differ from the east coast, although monsoon season is absent in the western coast of Peninsular Malaysia, (Benacer et al, 2016). In addition, the prevalence of leptospirosis in wildlife is positively correlated with humidity and temperature (Astudillo et al., 2012).

5.2 Brucellosis

The RBPT is a highly sensitive test and is the recommended method for herd/flock prevalence studies and as general purpose diagnostic test in all wildlife species. Absence of clinical signs but a positive serological test should be considered an infection (OIE, 2016). We did not find any seropositive samples for *B. abortus* and *B. melitensis* in this study. It is possible that the population is truly negative for the two species of the seroprevalence was too low to be detected by the sample size selected in this study. However, as the seroprevalence of brucellosis among the domestic animals are rather low at the animal-level (Anka et al, 2013; Bamaiyi, 2014), we believe that the seronegative finding is not unexpected as macaques may only contract the disease via ingesting materials contaminated with Brucella organism. As the organism is shed intermittently

and the organism does not persist for a very long time in the environment, the chances of contracting the organism by the macaques is minimized.

This finding can also be used to rule out the seroprevalence for other smooth type *Brucella sp.* such as *B. suis, B. neotomae, B. pinnipedialis, B. ceti, B. microti,* and *B. inopinata,* as they share O-polysaccharide which attaches to anti-Brucella lipopolysaccharide (LPS) antibodies detected by RBPT (Wareth et al., 2014; Al Dahouk et al., 2006). Our finding is similar to a sero-epidemiological study of free-ranging New World monkeys in Brazil (Molina et al., 2014).

This study have several limitations which should be taken into consideration in the interpretation of its findings. The use of existing serum sample from a collection pool from other project may result in information biases from uneven distribution of sampling. Samples were obtained from human-macaque conflict cases, therefore prevalence and risk factors of leptospirosis and brucellosis in this study cannot be used to conclude prevalence of these diseases in wild populations of primates at large. However, the findings suggest that macaques may posed a significant risk for cross-species transmission of leptospirosis to humans and other wildlife as demonstrated in some studies where infected primates may shed leptospires through intermittent leptospiruria (Baitchman et al., 2006; Szonyi et al., 2011). Thus, recognizing the need for more well-designed studies in the future for better understanding of the disease epidemiology in this species and management of its public health risk. The study may include more representative panel of MAT antigens and appropriate urine collection to identify the role of macaques as chronic carriers or reservoir.

The possibility of cross-species transmission of leptospirosis between macaques and humans calls for public education and change in human-macaque interactions to reduce this risk. Sustainable development and management of wildlife habitat is necessary to prevent displacement of wildlife. Change in human interaction with wildlife and displacement of wildlife from its habitat will cause change in disease dynamics and subsequently, emergence of infectious diseases. Surveillance of diseases in the wildlife population in the country will improve the management of infectious diseases and continue to enhance our understanding of the impacts of our actions to the environmental health, animal health and subsequently, human health.

CONCLUSION

We found 14% (14/100) seroprevalence of leptospirosis in long-tailed macaques with males being 4.5 times more likely to be infected than females. Our samples were seronegative for brucellosis in this study which suggests that macaques may not pose a significant public health risk of brucellosis as compared to leptospirosis. The infecting Leptospira sp. serovars are Cellodoni, Pyrogenes, Icterohaemorrhagiae, Bataviae and Lai. The discovery of serovar Cellodoni and Bataviae as infecting serovars of primates is new in Malaysia.

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APPENDICES

Figure 2a: Proportion of *Leptospira sp.* seropositive male and females long-tailed macaques.

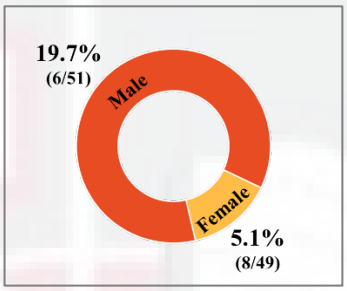


Figure 2b: Proportion of *Leptospira sp*. seropositive subadult and adult long-tailed macaques.

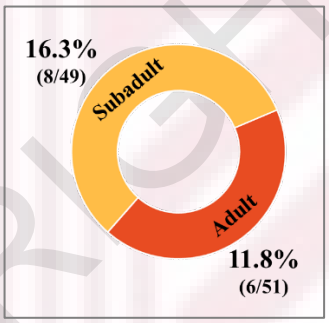
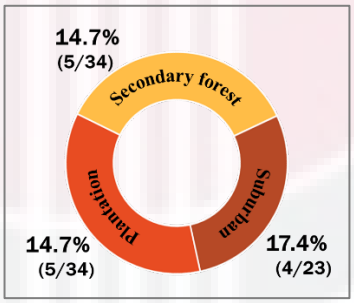
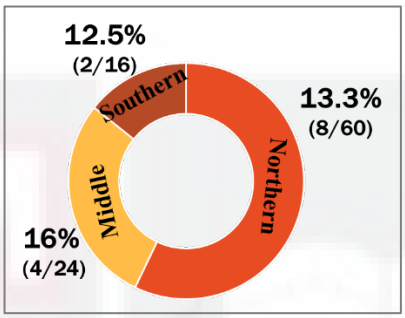


Figure 2c: Proportion of *Leptospira sp*. seropositive across habitats of long-tailed macaques.



*Urban habitat: 0 % (0/9)

Figure 2d: Proportion of *Leptospira sp.* seropositivity across region.



APPENDICES

Table 1: Long-tailed macaques data extracted and used in this study.

No.	Sex	Age	Habitat	Region	Location
1.	Male	Adult	Secondary Forest	Middle	Selangor
2.	Male	Subadult	Suburban	Middle	Pahang
3.	Male	Adult	Secondary Forest	Middle	Pahang
4.	Male	Adult	Secondary Forest	Middle	Kelantan
5.	Female	Subadult	Plantation	Northern	Kelantan
6.	Female	Adult	Plantation	Northern	Kelantan
7.	Male	Subadult	Plantation	Northern	Kelantan
8.	Male	Subadult	Plantation	Northern	Kelantan
9.	Female	Adult	Plantation	Northern	Kelantan
10.	Male	Adult	Suburban	Northern	Kelantan
11.	Male	Adult	Suburban	Northern	Kelantan
12.	Female	Adult	Suburban	Northern	Kelantan
13.	Female	Adult	Suburban	Northern	Kelantan
14.	Male	Subadult	Urban	Northern	Perlis
15.	Male	Subadult	Urban	Northern	Perlis
16.	Male	Subadult	Urban	Northern	Perlis
17.	Male	Adult	Suburban	Northern	Perlis
18.	Male	Adult	Secondary Forest	Northern	Perlis
19.	Male	Subadult	Secondary Forest	Northern	Perlis
20.	Male	Subadult	Secondary Forest	Northern	Perlis
21.	Male	Adult	Secondary Forest	Northern	Perlis
22.	Male	Adult	Secondary Forest	Northern	Perlis
23.	Female	Adult	Secondary Forest	Northern	Perlis
24.	Male	Adult	Secondary Forest	Northern	Perlis
25.	Female	Subadult	Secondary Forest	Northern	Perlis
26.	Male	Adult	Secondary Forest	Northern	Perlis
27.	Female	Adult	Secondary Forest	Middle	Pahang
28.	Male	Subadult	Urban	Middle	Pahang
29.	Female	Adult	Suburban	Southern	Pahang
30.	Male	Subadult	Suburban	Southern	Pahang
31.	Male	Subadult	Suburban	Southern	Pahang
32.	Male	Subadult	Suburban	Southern	Pahang
33.	Female	Adult	Urban	Middle	Kelantan
34.	Female	Adult	Urban	Middle	Kelantan
35.	Female	Adult	Urban	Middle	Kelantan
36.	Male	Adult	Plantation	Northern	Kelantan
37.	Female	Adult	Plantation	Northern	Kelantan
38.	Male	Adult	Plantation	Northern	Kedah
39.	Female	Adult	Plantation	Northern	Kedah
40.	Male	Subadult	Secondary Forest	Northern	Kedah
41.	Male	Subadult	Plantation	Northern	Kedah
42.	Male	Subadult	Plantation	Northern	Kedah
43.	Female	Adult	Secondary Forest	Northern	Kedah
44.	Female	Adult	Secondary Forest	Northern	Kedah
45.	Male	Subadult	Urban	Northern	Kedah
46.	Male	Adult	Plantation	Northern	Perlis
47.	Female	Adult	Plantation	Northern	Perlis
48.	Female	Adult	Secondary Forest	Northern	Perlis
49.	Female	Adult	Secondary Forest	Northern	Perlis
50.	Male	Adult	Plantation	Northern	Kedah
51.	Male	Subadult	Plantation	Northern	Kedah
52.	Female	Adult	Suburban	Northern	Kedah
53.	Female	Subadult	Suburban	Northern	Kedah
54.	Female	Subadult	Suburban	Northern	Kedah
55.	Female	Adult	Suburban	Northern	Kedah
56.	Male	Subadult	Suburban	Northern	Kelantan

57.	Male	Adult	Urban	Northern	Kedah
58.	Female	Adult	Secondary Forest	Northern	Perlis
59.	Female	Subadult	Secondary Forest	Northern	Kedah
60.	Female	Subadult	Secondary Forest	Northern	Kedah
61.	Female	Adult	Secondary Forest	Northern	Kedah
62.	Male	Subadult	Plantation	Northern	Kedah
63.	Male	Subadult	Plantation	Northern	Kedah
64.	Male	Subadult	Plantation	Northern	Kedah
65.	Male	Subadult	Plantation	Northern	Kedah
66.	Male	Subadult	Plantation	Northern	Kedah
67.	Male	Adult	Plantation	Northern	Kedah
68.	Male	Adult	Plantation	Northern	Kedah
69.	Female	Adult	Suburban	Northern	Kelantan
70.	Male	Subadult	Secondary Forest	Northern	Pahang
71.	Male	Subadult	Secondary Forest	Northern	Pahang
72.	Male	Subadult	Secondary Forest	Northern	Pahang
73.	Female	Adult	Plantation	Southern	Johor
74.	Male	Adult	Plantation	Southern	Johor
75.	Male	Subadult	Plantation	Southern	Johor
76.	Female	Adult	Secondary Forest	Middle	Kelantan
77.	Female	Adult	Suburban	Middle	Pahang
78.	Male	Adult	Suburban	Middle	Pahang
79.	Male	Subadult	Suburban	Middle	Pahang
80.	Male	Adult	Suburban	Middle	Pahang
81.	Male	Subadult	Suburban	Middle	Pahang
82.	Male	Adult	Suburban	Middle	Pahang
83.	Male	Adult	Suburban	Middle	Pahang
84.	Female	Subadult	Plantation	Southern	Johor
85.	Female	Adult	Plantation	Southern	Johor
86.	Female	Subadult	Plantation	Southern	Johor
87.	Female	Subadult	Plantation	Southern	Johor
88.	Female	Subadult	Plantation	Southern	Johor
89.	Male	Subadult	Plantation	Southern	Johor
90.	Male	Subadult	Plantation	Southern	Johor
91.	Female	Subadult	Plantation	Southern	Johor
92.	Male	Adult	Plantation	Southern	Johor
93.	Male	Subadult	Secondary Forest	Middle	Pahang
94.	Female	Subadult	Secondary Forest	Middle	Pahang
95.	Male	Subadult	Secondary Forest	Middle	Pahang
96.	Male	Subadult	Secondary Forest	Middle	Pahang
97.	Male	Subadult	Secondary Forest	Middle	Pahang
98.	Male	Subadult	Secondary Forest	Middle	Pahang
99.	Male	Subadult	Secondary Forest	Middle	Pahang
100.	Female	Adult	Secondary Forest	Northern	Kelantan